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Influence of Threonine Supplementation on Antioxidant Enzyme Activities and Haemato-biochemical Parameters of Commercial Layers in Sub-Tropics

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ABSRACT

A study was conducted to see the effects of threonine supplementation on antioxidant enzyme activities and haemato-biochemical parameters of commercial layers. Sixteen hundred and eighty (1680) BV-300 laying birds of 40 weeks age with an average body weight of 1353±5.86g were allocated in a completely randomized design with five (5) groups of 336 birds in each. Groups were formed according to the dose of supplemented threonine in various rations i.e. NRC specification, BV-300 strain requirement, 110% of BV-300 strain requirement, 120% of BV-300 strain requirement and 130% of BV-300 strain requirement. With the increasing levels of supplemental L-threonine, GSH-Px activity and serum SOD level increased linearly ($p=0.005$ and $p=0.002$, respectively). Among hematological parameters, though non-significant, Heterophils/Lymphocyte ratio tended to decrease linearly ($p=0.02$). The sugar and total protein concentration increased linearly ($p<0.001$) while albumin and globulin concentration increased linearly and quadratically. There was a linear decrease ($p<0.001$) in blood cholesterol level. It may be concluded that L-threonine supplementation at 130% of BV-300 recommendation has a better antioxidant function and better haemato-biochemical parameters.

Keywords: Anti-oxidant enzyme, GSH-Px, Catalase, H:L ratio, L-threonine

Poultry birds are not capable of synthesizing threonine *de novo*, the third limiting amino acid thereby making it nutritionally essential. Commercial L-threonine (98.5%) is generally added to the diet of birds in order to exactly match the dietary amino acid balance to the unique nutritional requirements of the poultry birds. The essential amino acids levels in the feed higher than NRC specifications are needed to achieve optimal growth performance, immune-competence and disease resistance (Quentin *et al*., 2005). NRC recommended levels were mainly examined in thermo-neutral environment, therefore, in tropical environment the effect of higher temperature should be considered during poultry ration formulation with recommended dose of amino acids. Summer season remains very hot and humid in subtropical countries with daily temperature averaging above 30°C with more than 75% relative humidity. The detrimental effects of high temperatures are exacerbated by high relative humidity (Romijn and Lokhorst, 1961) which ultimately leads to stress and reduced production in birds. As NRC requirement levels were mainly examined in thermo-neutral environment, therefore, the dietary threonine requirements as reported by the NRC (1994) may be insufficient for modern commercial layer strains reared under heat and relative heat stress. Moreover, threonine is one of the essential amino acids liable to be limited when high-humidity heat stress would decrease feed intake. During any stress condition, antioxidant enzyme activities and hemato-biochemical parameters mainly determine health status of birds. For example, heterophil/ lymphocyte ratio is a key indicator of the presence of physiological stress (Siegel, 1995). In the present experiment, the stress condition of layer birds in sub-tropical climate has been evaluated. Keeping the above facts in view, the present investigation was conducted to determine whether supplemental threonine

can positively influence antioxidant enzyme activities and haemato-biochemical parameters in commercial layers.

MATERIALS AND METHODS

Experimental Birds, experiment duration and climatic condition

Sixteen hundred and eighty (1680) BV-300 laying birds of 40 week of age (before the start of second phase of laying) with an average body weight of 1353±5.86g were distributed in a completely randomized design with five (5) groups of 336 birds in each (Table 1). Each treatment had four replicates of eighty four (84) birds. The experiment was carried out from the month of March to September, which is considered to be hot and humid period in West Bengal state. The temperature and humidity inside the shed were recorded twice daily (at 0800 and 1400 h). The average ambient RH inside the shed was 75 ± 10% and the mean daily temperature was 33 ± 8°C. The experiment lasted for 20 weeks.

Table 1: Allocation of different treatment groups and threonine level for BV-300 Laying Hens

Group	Threonine levels	Layer (40-60 wks)	Thr/Thr
T1	Basal diet without supplemented threonine, (100% of NRC'1994 specification)	0.47	0.59
T2	Basal diet with supplemented threonine (100% of BV-300 strain Requirement)	0.52	0.65
T3	110% of BV-300 strain requirement	0.57	0.71
T4	120% of BV-300 strain requirement	0.62	0.78
T5	130% of BV-300 strain requirement	0.68	0.85

Housing, light and diets

All the experimental layer birds were maintained in a cage system layer house with standard hygienic condition following all bio-security measures throughout the experimental period. The layers were housed in metal wire cages and kept individually and the size of each cage was 24″ wide × 25.125″ deep × 14″ high. The light programme used for layer was maintained at 17 hours of daylight and 7 hours of darkness throughout the studies. Layers were fed on the basal diet based on maize, Soybean meal, wheat bran groundnut cake, oil, minerals, vitamins, premixes and crystalline amino acids. Feed in mash form and water were provided freely. Crystalline L-threonine (98.5% Threonine, Evonic, Singapore) was added to the basal diet at various percentages. Prior to feed formulation, individual feed ingredients were analyzed for amino acids by HPLC. All the mash rations were also analyzed for respective amino acid content. To determine the nutritional levels of threonine, iso-nitrogenous and iso-caloric diets were formulated by supplementing L-glutamic acid in the premix (Table 2). Feed in mash form and wholesome and clean drinking water was provided *ad libitum* to all experimental birds throughout the experimental period. Layers were fed on the same basal diet based on maize, Soybean meal, groundnut cake, wheat bran, oil, minerals, vitamins, salts & premixes and crystalline amino acids. The pooled feed samples were subjected for analysis of dry matter (DM), organic matter (OM), crude protein (CP), crude fibre (CF), nitrogen free extract (NFE) and ether extract (EE), Ca and P as per AOAC, 1995. Vaccination was performed in against Newcastle disease on 50th week by Lasota strain.

Table 2: Composition of basal diets and chemical analysis (fulfilling 100% threonine requirement as per NRC'1994 without added threonine)

Ingredients	Layer (40-60 wks)
Yellow maize	570
Soybean meal	36
Groundnut Cake (SE)	170
Wheat Bran	103
Oil	0
DCP	10
Limestone	100
Iodized Salt	2
L-Lysine HCl	1.3
DL-Methionine	1.5
Pre-mix*	6.2
Nutrient content DMB (%)	
CP	17.52
EE	3.19
TA	9.43
AIA	1.91
Crude fibre	7.44
Calcium	4.05

Total Phosphorus	0.87
Lysine	0.80
Methionine	0.40
Cystine	0.352
Methionine and Cystine	0.752
Arginine	0.84
Tryptophan	0.18
Threonine	0.47
Calculated ME (Kcal/Kg)	2558

Premix provided the following per kilogram: vitamin A, 7,000 IU; vitamin D3, 2,500 IU; vitamin E, 36 mg; vitamin K, 32 mg; vitamin B1, 2 mg; vitamin B2, 5.6 mg; vitamin B6, 4 mg; vitamin B12, 0.025 mg; nicotinic acid, 38 mg; folic acid, 1.1 mg; calcium pantothenate, 10 mg; biotin, 0.16 mg; Cu, 10 mg; Fe, 80 mg; Mn, 100 mg; Zn, 60 mg; I, 0.55 mg; Se, 0.12 mg.

Other premixes**:** Phytase 5000, choline chloride, toxin binder, cocktail enzyme, coccidiostat, emulsifier, antioxidant, sodium bi-carbonate, liver tonic and L-glutamic acid.

Collection and analysis of blood

Representative blood samples (about 5ml) were collected at 60th week from the wing veins of the layer birds. Blood samples were placed in the centrifuge tube without anticoagulant (20 birds/ treatment, total 100 birds). Centrifuge tubes were placed in refrigerator at 4^{o}C. Serum was harvested by centrifuging the whole blood at 2500 rpm for 15 minutes and stored in deep freeze at -80^{o}C for further analysis. The haematological parameters were estimated by standard methods described by Jain, 1993. The serum biochemical parameters were carried out with reagents and procedures supplied along with the kits (Trans Asia Bio-Medicals Ltd., Solan, HP, India). For anti-oxidant enzyme study, the serums were subjected to the measurement of SOD, GSH-Px, and catalase concentrations by spectrophotometric methods using a spectrophotometer (UV-2000, Unico Instruments Co. Ltd., Shanghai, China). All of the assays were done by chemical methods. Activity of SOD was measured by the epinephrine method at 480 nm, which monitors the inhibition of reduction of nitro blue tetrazolium by the sample (Misera and Fridovich, 1972). Activity of GSH-Px was detected with 5,50-dithiobis-p-nitrobenzoic acid, and the change of absorbance at 412nm was monitored using a spectrophotometer (Griffith, 1980). Catalase activity was done as per Beers and Sizer, 1952 and the absorbance was recorded at 240nm.

Statistical analysis

The data were subjected to one way analysis of variance (Snedecor and Cochran, 1994) in the Statistical Package for Social Sciences, 2002 (SPSS 21.0, Chicago, IL, USA). Whenever significant differences were found (at $P<0.05$), the treatment means were compared using Tukey's test. The various dependent parameters were also studied for linear and quadratic responses using polygonal contrasts.

RESULTS AND DISCUSSION

Effect of threonine on anti-oxidant enzyme

Anti-oxidant enzymes like SOD, catalase and GSH-Px are the main anti-oxidant parameters used to assess oxidative status in the enzymatic system. Group with 130% of BV-300 requirement had higher ($p<0.05$) GSH-Px concentration than the NRC specification group (Table 3). GSH-Px also linearly increased ($p=0.005$) with the increasing levels of

Table 3: The Effects of Supplemental L-threonine on Serum Antioxidant Enzyme Activities of BV-300 Laying Hens (40-60 weeks)

Parameters	NRC specification	BV 300 requirement	110% of BV 300 requirement	120% of BV 300 requirement	130% of BV 300 requirement	SEM	P-value	Linear	Quadratic
Glutathione Peroxidase (µmol/L)	517.14^{a}	585.31^{ab}	597.15^{ab}	696.19^{ab}	738.66^{b}	53.81	0.043	0.005	0.87
SOD (U/mL)	130.70^{a}	132.57^{a}	136.21^{a}	134.52^{a}	143.09^{b}	2.90	0.01	0.002	0.34
Catalase (Unit/ mL)	16.25	16.95	16.93	18.93	18.58	0.78	0.12	0.02	0.98

Means bearing different superscripts in the same row differ significantly ($p<0.05$)

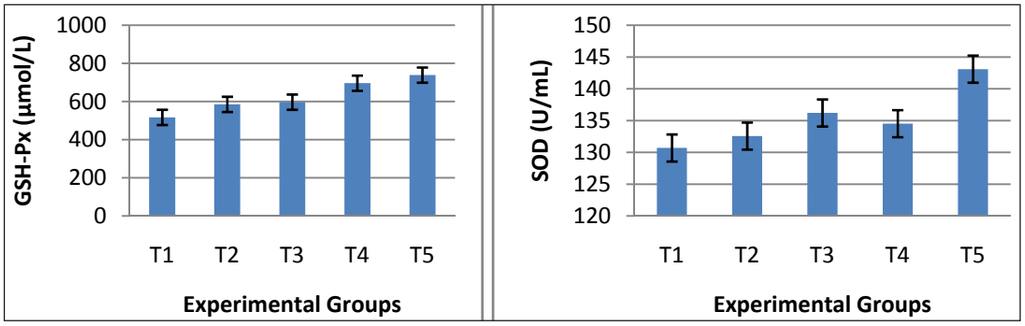

Fig. 1: The effect of supplemental threonine on Glutathione per-oxidase and SOD level in the experimental layer birds

supplemental threonine level in diet. Group with 130% of BV-300 requirement showed higher concentration ($p<0.05$) of SOD than rest of the experimental groups. The serum SOD level also linearly increased ($p=0.002$) with the increasing levels of supplemental threonine level (Fig. 1).

High temperature can disturb the balance between the production of reactive oxygen species (ROS) and the antioxidant system which may further stimulate the formation of ROS (Lin *et al*., 2008; Feng *et al*., 2008). Habte-Tsion *et al.* (2016) observed that glutathione peroxidase (GPx) increased with increasing dietary threonine levels from 1.58% to 2.08% in juvenile blunt snout bream. Beneficial effect noticed in the treatment groups might be due to an increased mRNA level on increased concentration of threonine supplementation which might have up-regulated the gene transcription of antioxidant enzymes GSH-Px (Habte-Tsion *et al.,* 2016). Therefore, adding threonine may maximize the concentration of GSH-Px in the serum and may protect cells of layer birds from oxidative injury by clearing superoxide anions

Azzam *et al.* (2012) (at 0.47, 0.66 and 0.74% threonine) revealed that threonine supplementation in broiler birds had significant effect ($p<0.05$) on serum SOD activity. Threonine is one of the amino acids that are able to carry a small fraction of copper (Cu) in the blood (Shils *et al*., 2006). Copper-zinc superoxide dismutase is known to have oxidation-retarding factor (Meyer *et al.,* 1994). The SOD is one of the main antioxidant enzymes in scavenging the oxygen free radicals (McCord, 1979). One of the important strategies in poultry farms during the summer months is to maximize antioxidant ability and minimize lipid peroxidation. Secondly, better effect in the threonine supplemented group might be due to increased mRNA level on increased concentration of threonine supplementation which might have up-regulated the gene transcription of antioxidant enzymes gene expressions of Cu-SOD (Habte-Tsion *et al.,* 2016). Therefore, adding threonine might have maximized the concentration of SOD in the serum and may protect cells from oxidative injury by clearing superoxide anions.

Effect of threonine on haematological parameters

The hemoglobin and values of haematocrit level of blood among the experimental groups of layers were not significant ($p>0.05$) at increased levels of L-threonine supplementation (Table 4). The values of MCV, MCH (pg) and MCHC percentage of blood did not differ ($p>0.05$) among the experimental layers. The values of TEC (million/cc) and TLC (thousand/cc) did not differ ($p>0.05$) among the experimental layer birds. The values of heterophils percentage did not differ ($p>0.05$) among the experimental layer birds. However, the average heterophils percentage tended to decrease linearly ($p=0.07$). The average lymphocyte percentage increased linearly ($p=0.003$) with the increasing levels of L-threonine in the diet. Though, Heterophils/Lymphocyte ratio did not differ ($p>0.05$) among the experimental layers, however, H,L ratio tended to decrease linearly ($p=0.02$) with the increasing levels of supplemental threonine.

Rezeipour *et al.* 2012 (at 0, 0.25, 0.5 and 0.75% threonine) also observed that the levels of threonine did not affect the hematological profile (haemoglobin and hematocrit value). Present observation revealed that layer birds

Table 4: The Effects of Supplemental L-threonine on Hematological Parameters of BV-300 Laying Hens (40-60 weeks)

Parameters	NRC specification	BV 300 requirement	110% of BV 300 requirement	120% of BV 300 requirement	130% of BV 300 requirement	SEM	P- value	Linear	Quadratic
Hb (%)	9.28	9.30	9.23	9.18	9.22	0.13	0.97	0.60	0.88
Haematocrit (%)	29.00	29.25	28.50	27.63	27.75	1.29	0.86	0.33	0.94
MCV (fL)	124.10	123.68	125.08	125.71	125.79	2.45	0.96	0.49	0.99
MCH (pg)	39.78	39.50	40.76	41.82	41.85	1.10	0.43	0.09	0.92
MCHC (%)	32.03	32.02	32.63	33.34	33.30	1.05	0.82	0.27	0.99
TEC (10^6/cc)	2.35	2.36	2.28	2.20	2.21	0.09	0.59	0.14	1.00
TLC (000/cc)	21.85	22.10	22.55	22.48	22.60	0.45	0.73	0.22	0.66
Heterophils (%)	28.00	28.25	28.00	27.75	27.00	0.40	0.26	0.07	0.20
Lymphocyte (%)	66.75^{a}	66.75^{a}	67.75^{ab}	67.50^{ab}	69.00^{b}	0.47	0.02	0.003	0.34
Heterophils: Lymphocyte	0.42	0.43	0.41	0.41	0.39	0.002	0.09	0.02	0.22

Means bearing different superscripts in the same row differ significantly ($p<0.05$).

with higher levels of supplemental L-threonine tended to lower heterophils percentage; however, it could not be established statistically. The heterophils/ lymphocyte (H/L ratio) appears to be more reliable indicator of stress and higher value of H/L means the birds are in more stressful condition (Gross and Siegal, 1983). In the majority of avian species, healthy animals have more lymphocytes than heterophils in circulation, which influences the H/L ratio. Da silva *et al*. (2010) also concluded that in stress condition, the corticoids when released in the blood, decreases the number of lymphocytes. Heshmat *et al.,* 2013 (at 0.80, 0.87, 0.94 and 1.01% threonine) also revealed that the levels of threonine in the diet did not affect the H/L ratio. Nevertheless, specific mechanisms regarding the effect of threonine on the blood hematology in birds need further study.

Effect of threonine on biochemical parameters

With the increasing levels of supplemental L-threonine in the diet, the average serum glucose concentration was higher ($p<0.05$) in group with 120% of BV-300 requirement when compared either with NRC specification or BV 300 recommended group (Table. 5). The serum glucose concentration also linearly increased ($p<0.001$). There was a linear increase ($p<0.001$) in serum total protein level. The serum albumin level varied both linearly ($p=0.002$) and quadratically ($p=0.02$). The average serum globulin level was higher ($p<0.001$) in 130% of BV-300 requirement group than rest of the groups. There was a linear ($p<0.001$) and quadratic ($p=0.03$) increment in serum globulin level. Lower ($p<0.05$) concentration of serum cholesterol level was observed in group having 120% and 130% threonine as compared to BV-300 requirement group (Fig. 2). There was a linear decrease ($p=0.002$) in cholesterol level with the increasing levels of supplemental L-threonine.

Weber *et al.* (2013) revealed that serum glucose concentrations linearly increased ($p<0.002$) with increasing levels of L-threonine. Abdel-Wareth and Esmail, 2014 (at 0.47, 0.67, 0.87, 1.07% threonine) also revealed that serum glucose concentration significantly increased ($p<0.002$) with increased L-threonine supplementation. High concentration of serum glucose in the L-threonine supplemented groups might be due to catabolism of L-threonine in body. Catabolism results in glucogenic products like pyruvate and propionate that are needed for energy or glucose production. Poultry birds use threonine with high efficiency for protein accretion when dietary threonine is at or below the requirement whereas they use this amino acid as a glucogenic precursor in excess amount (Edwards *et al*., 1997). These biochemical processes are responsible for increased serum glucose level in the treatment groups.

The present investigation is supported by Azzam *et al.*, 2011 (at 0.47, 0.57, 0.67, 0.77 and 0.87% threonine)

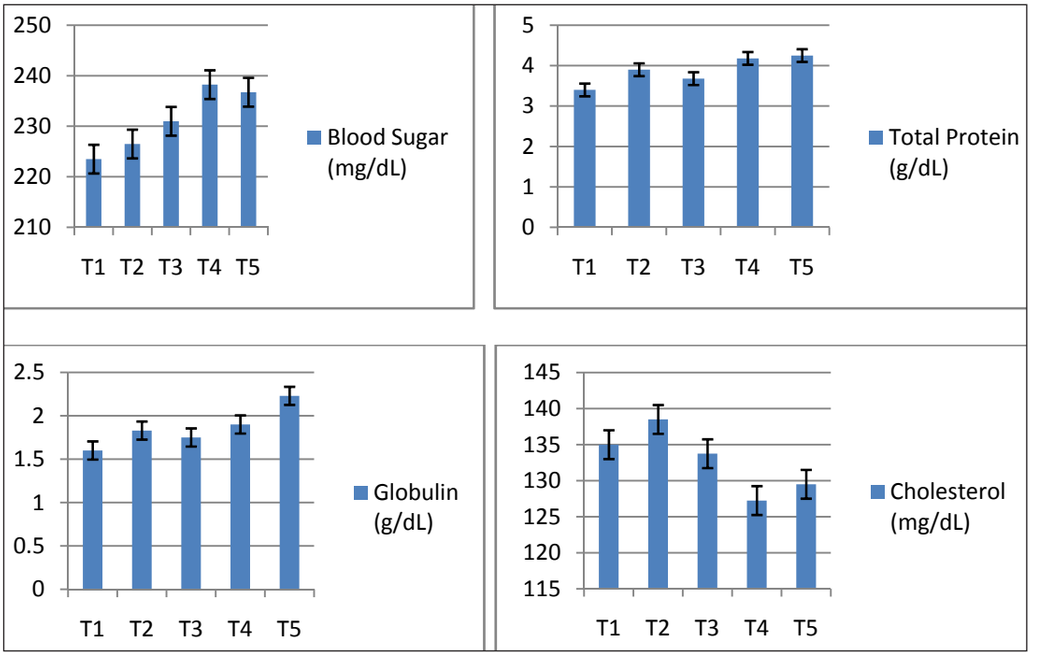

Fig. 2: The effect of supplemental threonine on blood sugar, total proteins, globulin and cholesterol level in the experimental layer birds

Table 5: The Effects of Supplemental L-threonine on Serum Biochemical Parameters of BV-300 Laying Hens (40-60 weeks)

Parameters	NRC specification	BV 300 requirement	110% of BV 300 requirement	120% of BV 300 requirement	130% of BV 300 requirement	SEM	P value	Linear	Quadratic
Blood Sugar (mg/dL)	223.50^{a}	226.50^{ab}	231.00^{abc}	238.25^{c}	236.75^{bc}	2.35	0.002	<0.001	0.49
Uric Acid (mg/dL)	4.18	4.23	4.05	4.03	4.00	0.09	0.33	0.07	1.00
Creatinine (mg/dL)	0.75	0.80	0.70	0.73	0.68	0.05	0.53	0.20	0.71
Total Protein (g/dL)	3.4^{a}	3.9^{bc}	3.68^{ab}	4.18^{c}	4.25^{c}	0.09	<0.001	<0.001	0.70
Albumin (g/dL)	1.80^{a}	2.08^{bc}	1.93^{ab}	2.28^{c}	2.03^{ab}	0.05	<0.001	0.002	0.02
Globulin (g/dL)	1.6^{a}	1.83^{b}	1.75^{ab}	1.90^{b}	2.23^{c}	0.04	<0.001	<0.001	0.03
Albumin: Globulin	1.13	1.14	1.10	1.07	1.03	0.027	0.06	0.007	0.28
ALT (IU/L)	18.50	16.75	17.50	17.75	18.25	1.06	0.80	0.88	0.33
AST (IU/L)	238.75	240.25	234.75	236.00	230.25	4.30	0.54	0.14	0.64
ALP (IU/L)	172.5	149.5	158.25	155.5	167.00	5.73	0.08	0.79	0.02
TGL (mg/dl)	143.5	134.5	141.00	151.75	137.75	3.97	0.07	0.65	0.70
Cholesterol (mg/dL)	135^{ab}	138.5^{b}	133.75^{ab}	127.25^{a}	129.5^{a}	1.83	0.004	0.002	0.55
HDL (mg/dL)	99.00	101.75	99.25	95.25	96.25	1.52	0.06	0.03	0.39
LDL (mg/dL)	23.50	25.25	23.25	22.25	22.75	0.77	0.12	0.09	0.61
VLDL (mg/dL)	12.50	11.50	11.25	9.75	10.50	0.94	0.34	0.07	0.53
Ca (mg/dL)	16.33	16.18	16.05	16.55	16.35	0.24	0.64	0.58	0.56
P (mg/dL)	10.15	9.90	10.08	10.03	10.10	0.07	0.21	0.92	0.14

Means bearing different superscripts in the same row differ significantly ($p<0.05$).

who revealed that serum total protein concentration quadratically increased to supplemental L-threonine with a maximum response at 0.67 and 0.77% supplemental L-threonine at 48 weeks of age in laying hens. In the process of protein anabolism and proteolysis, the serum protein level is always an indicator of the protein metabolism and immunity function situation *in vivo*. Avian total protein contains albumin and α, β and γ-globulin (Lumeij, 1997). High concentrations of total protein are associated with significant increases in levels of serum albumin and globulin (Hunt and Hunsaker, 1965).

Azzam and El-Gogary, 2015 (at 0.69, 0.71, 0.74, 0.76 and 0.79% threonine) revealed that serum cholesterol decreased quadratically ($p<0.05$) in broilers with supplemental L-threonine levels. The lowering of cholesterol level by supplemental threonine might be due to production of more amounts of bile acid/bile salts in the liver. Twenty percent (20%) of excess threonine is converted to glycine which in turn synthesizes bile acids. Bile acids are synthesized from cholesterol, a major constituent of cholesterol metabolism. Hence, bile acids provide major excretory route of cholesterol. Bile acid sequestrants bind bile acids and prevent re-absorption of bile acids and hence cholesterol is lost in faeces. Hence, there will be disruption of enterohepatic circulation of bile acids and it will lower blood cholesterol level. Rezaeipour and Gazani (2014) also revealed that serum VLDL levels were reduced by dietary L-threonine supplementation ($p<0.05$).

CONCLUSION

The present study assessed the effects of supplemental threonine on antioxidant enzyme activity and haemato-biochemical parameters of commercial layers in the sub-tropics. From the results it may be concluded that L-threonine supplementation at 30% higher than BV-300 requirement level has a better antioxidant function, better haemato-biochemical parameters under the subtropical climate.

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